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14. ABSTRACT

The aim of this project is to characterize the functional significance of $p57_{Kip2}$, one of Cyclin-dependent kinases inhibitors (CKI) of the INK4 family, in prostate proliferation, differentiation, tumorigenesis, and progression. In the present study, we have investigated the expression of $p57_{Kip2}$ in human prostate cancer cases by immunohistochemistry. The average $p57_{Kip2}$ labeling index in noncancerous lesions was 47.47%. However, the labeling index significantly decreased ($p<0.001$) in PIN (10.21%) and carcinoma (2.85%) lesions. When virus-mediated overexpression of $p57_{Kip2}$ in prostate cancer cells (LNCaP), significantly suppressed the cells' motility, potential for invasion, arrested the cell cycles at G0/G1 stage, and induced apoptosis. Furthermore, when the LNCaP cells stable transfected by $p57_{Kip2}$expression vector were recombined with rat urogenital mesenchyme (rUGM) and subsequently grafted into a male athymic mouse host using tissue recombinant techniques, the LNCaP tumors transformed into well differentiated squamous tumors and showed increased keratin synthesis or no tumor formation in athymic mice. These results suggest that decreased expression of $p57_{Kip2}$ occurs frequently in human prostate cancer even early in PIN lesion and $p57_{Kip2}$ overexpression contributes to the downregulation of cell proliferation. Thus, $p57_{Kip2}$ is an important gene in prostate cancer tumorigenesis and progression.

15. SUBJECT TERMS
Prostate cancer, $p57^{Kip2}$, tissue recombination

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Statement of Work

Investigating the Role of $p57^{Kip2}$ in Prostate Cancer

Task 1. Characterize the $p57^{Kip2}$ expression in the human prostate cancer. (months 1-6):

1. Determine the levels of expression of $p57^{Kip2}$ in human prostate cancer by Immunohistochemical staining. (months 1-3).
2. Confirm the expression pattern of $p57^{Kip2}$ in human prostate cancer by *in situ* hybridization and/or RT-PCR. (months 3-6).
3. Statistical analysis. (months 5-6).

Task 2. Determine the in vivo functional role of $p57^{Kip2}$ in prostate development and tumorigenesis using tissue recombination techniques. (months 6-12):

1. Rescue and tissue recombination will be performed using different genotypes [$p57^{Kip2\ (-/-)}$, $p57^{Kip2\ (+/-)}$, $p57^{Kip2\ (+/+)}$] of urogenital tissues from $p57^{Kip2}$ knockout mice. Rescued tissues will be harvest at 4-6 weeks for histological and molecular biological analysis. Epithelial cells from rescued tissues will be collected and grafted underneath the renal capsules of nude mice host with rat urogenital mesenchyme (rUGM) using tissue recombinant techniques. Tissue samples will be collected at 1, 2 and 3 month after grafting. Ten mice per group are required. (months 6-12)
2. Characterize grafted rescued and recombined prostate epithelial tissues. (months 6-12).
   a. Histological characterization.
   b. Western blot and Immunohistochemical analysis
   c. Proliferation and apoptosis quantification.
   d. Hormonal dependence.

Task 3. Investigate the functional role of $p57^{Kip2}$ in prostate cancer cellular processes. (months 12-24):

1. Construct a $p57^{Kip2}$ expression vector using tetracycline-inducible system and establish stable prostate cancer cell lines of LNCaP, PC-3 which produce tetracycline repressor protein for investigation. (months 12-15)
2. Investigate the functional role of $p57^{Kip2}$ in cellular processes using the $p57^{Kip2}$ expression vector transfected/infected into prostate cancer cell lines. (months 12-24)
   a. Cell cycle and Apoptosis analysis (Flow Cytometric analysis)
   b. Proliferation (Brdu labeling, MTT assay)
   c. Characterize the functional role and mechanism of $p57^{Kip2}$ in prostate cancer cellular processes (Western Blot, Immunoprecipitation, and CDKs activity assay etc.)
3. Recombine Tet-inducible $p57^{Kip2}$ stable transfected prostate cancer cells (using LNCaP cells) with rUGM cells to perform tissue recombinant experiments. The $p57^{Kip2}$ stable transfected cells recombinants will be grafted underneath the renal capsules of male athymic mice. Either Dox will be added at 1 month after establishing the LNCaP tumors or controls will remain untreated. Recombinant tissues will be collected at 1, 2 and 3 month after grafting. However, depending upon the rate of tumor growth and histological changes, we will decide to turn on and turn off the $p57^{Kip2}$ expression at a given time point to look at short term and long term changes compared to controls. Ten mice per group are required. (months 12-24)
   a. Histological characterization.
   b. Western blot and Immunohistochemical analysis
   c. Proliferation and apoptosis quantification.

Introduction:

Aberrations in the normal cycling of a cell lead to uncontrolled proliferation and can result in the development of cancer. $p57^{Kip2}$, one of Cyclin-dependent kinases inhibitors (CKI) of the INK4 family, is located on human chromosome 11p15.5, a region implicated in sporadic cancers. Because of its location, biochemical activities, and imprinting status, $p57^{Kip2}$ has been considered a candidate tumor suppressor gene. However, the mechanism by which $p57^{Kip2}$ exerts its modulatory functions in prostate differentiation and tumorigenesis/progression is not yet fully understood.

The goal of this project is to characterize the functional significance of $p57^{Kip2}$ in prostate proliferation, differentiation, tumorigenesis, and progression. The central hypothesis of this proposal is that altered expression of $p57^{Kip2}$ is important in development and/or progression of prostate adenocarcinoma.

To understand the role of $p57^{Kip2}$ in prostate cancer, we have investigated the expression of $p57^{Kip2}$ in 42 human prostate cancer cases by immunohistochemistry. The average $p57^{Kip2}$ labeling index in noncancerous lesions was 47.47%. However, the labeling index significantly decreased ($p<0.001$) in PIN (10.21%) and carcinoma (2.85%) lesions. To further understanding the role of $p57^{Kip2}$ on the prostate cancer progression, we investigated the effects of $p57^{Kip2}$ on the prostate cancer cells *in vitro*. When virus-mediated overexpression of $p57^{Kip2}$ in prostate cancer cells (LNCaP), significantly suppressed the cells' motility, potential for invasion, arrested the cell cycles at G0/G1 stage, and induced apoptosis. Furthermore, when the LNCaP cells stable tansfected by $p57^{Kip2}$ expression vector were recombined with rUGM and subsequently grafted into a male athymic mouse host using tissue recombinant techniques, the LNCaP tumors transformed into well-differentiated squamous tumors and showed increased keratin synthesis or no tumor formation in nude mice.

These results suggest that decreased expression of $p57^{Kip2}$ occurs frequently in human prostate cancer even early in PIN lesion and $p57^{Kip2}$ overexpression contributes to the downregulation of cell proliferation. Thus, $p57^{Kip2}$ is an important gene in prostate cancer tumorigenesis and progression.

To further understanding the role of $p57^{Kip2}$ on the prostate development and cancer progression, we already acquired the $p57^{Kip2}$ knockout mice and currently actively breading and maintain this line in our laboratory. The prostates of $p57^{Kip2}$ knockout mice will be grafted beneath the renal capsule of the male athymic mice to monitor prostatic development, tumorigenesis, and prostate cancer progression.

Key research accomplishments

Task 1. Characterize the $p57^{Kip2}$ expression in the human prostate cancer.

- We have investigated the expression of $p57^{Kip2}$ in 42 human prostate cancer cases by immunohistochemistry (**Fig. 1**).
- Two pathologists from Vanderbilt University Medical Center graded and counted all specimens in blinded fashion. Cells were counted as positive for $p57^{Kip2}$ when immunoreactivity is clearly observed in their nuclei. We counted positive cells for $p57^{Kip2}$ by monitoring at least 200 cells for noncancerous, PIN and carcinoma lesions in multiple regions of the same sample.
- The results (labeling index) were analyzed using Newman-Keuls test (q-test), with significance defined as $P < 0.05$ (**Fig. 2**).
- We will use fresh human prostate cancer samples to confirm the expression pattern of $p57^{Kip2}$ in human prostate cancer by *in situ* hybridization and/or RT-PCR. We have been collecting sample via the Vanderbilt-Ingram Cancer Center repository. When sufficient samples are collected, we will isolate RNA at the same time and perform RT-PCR. For *in situ* hybridization studies, the tissue must be fixed by a different procedure. This is being done. We are moving forward at a faster pace with Task 3.

Fig.1.

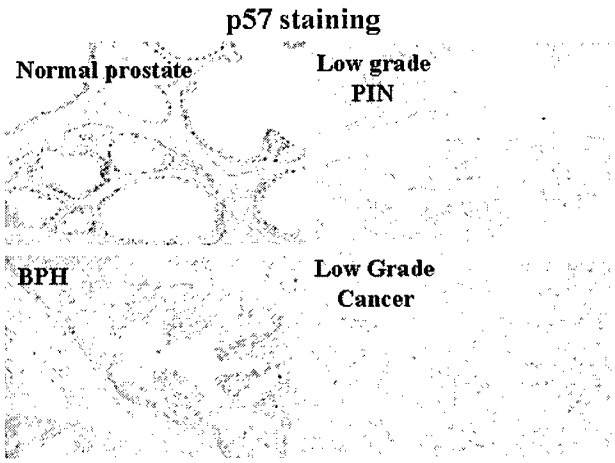

Fig.2.

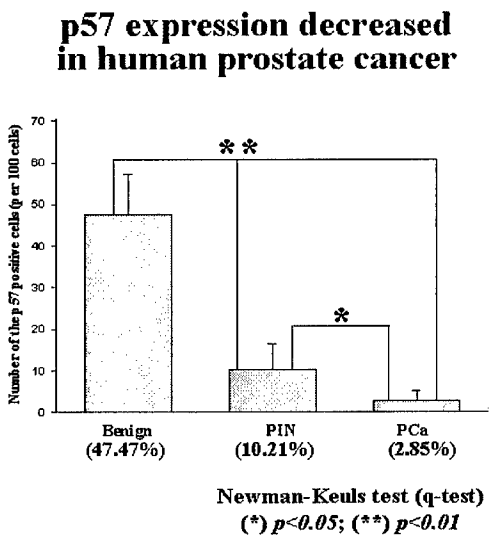

Fig. 1, 2: Immunohistochemical detection of $p57^{Kip2}$ expression in human prostate cancer (Fig. 1). The average $p57^{Kip2}$ labeling index in noncancerous lesions (BPH) was 47.47%. However, the labeling index significantly decreased ($p<0.001$) (Newman-Keuls q-test) in PIN (10.21%) and carcinoma (2.85%) lesions (Fig. 2).

Task 2. Determine the in vivo functional role of $p57^{Kip2}$ in prostate development and tumorigenesis using tissue recombination techniques.

- We already acquired the $p57^{Kip2}$ knockout mice and currently actively breading and maintain this line in our laboratory. After receiving the first heterozygotic male from Dr. Pumin Zhang at Baylor College of Medicine, we started the breeding program. Unfortunately, this male would not breed. A new shipment of mice was send from Dr. Pumin Zhang; however, it was delayed until he had breed enough of mice that he could spare some to ship us. As a result, we are first now trying to breed the mice for the experiments outlined in this task.

- The prostates of $p57^{Kip2}$ knockout mice will be grafted beneath the renal capsule of the male athymic mice to monitor prostatic development, tumorigenesis, and prostate cancer progression as described in the SOW.

Task 3. Investigate the functional role of $p57^{Kip2}$ in prostate cancer cellular processes.

Although we are behind schedule in Task 1 as we still collect samples and we could not start Task 2 since the first $p57^{Kip2}$ knockout mice would not breed, we have been able to move ahead of schedule with Task 3.

- We have constructed a $p57^{Kip2}$ expression vector using tetracycline-inducible system.
- TetR expressing LNCaP cells were kindly provided by Susan Logan (NYU School of Medicine at VAMC).
- We have established Tet-inducible $p57^{Kip2}$ stable transfected prostate cancer cell line using TetR expressing LNCaP cells (**Fig. 3**).

Fig.3.

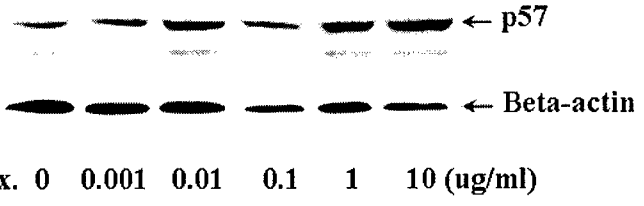

Fig. 3: Establishing the Tet-inducible $p57^{Kip2}$ stable transfected prostate cancer cells. We constructed a $p57^{Kip2}$ expression vector using tetracycline-inducible system and infected that to the TetR expressing LNCaP cells. Established LNCaP-p57 cells response to the Doxycycline dose dependently.

- We have investigated the functional role of $p57^{Kip2}$ in cell motility and invasion by Transwell Invasion Assay (**Fig. 4**). In addition, we have investigated the affect of $p57^{Kip2}$ on cell cycles and apoptosis by Flow Cytometric Analysis (**Fig. 5**).

Fig. 4.

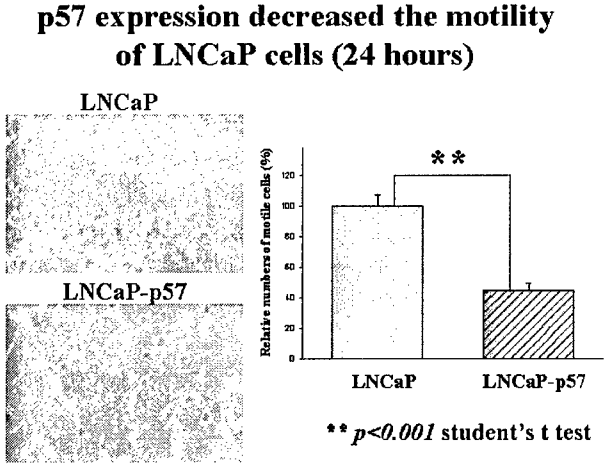

Fig. 5.

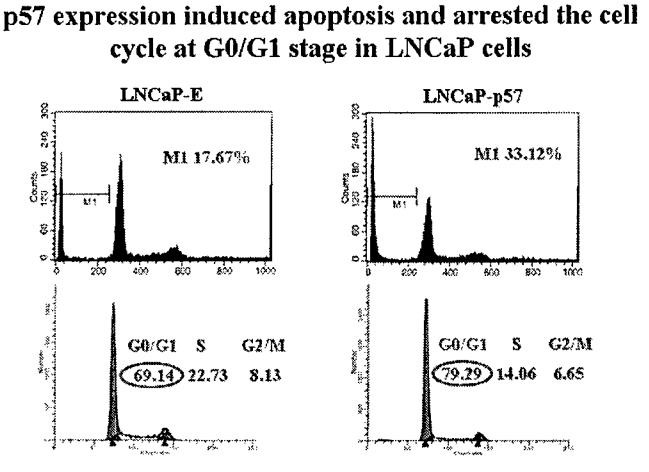

Fig. 4, 5: Transwell Invasion Assay and Flow Cytometric Analysis investigated the functional role of p57Kip2 in prostate cancer cellular processes. Overexpression of $p57^{Kip2}$ reduced the motility and

invasion of LNCaP cells *in vitro* (Fig. 4), but also arrested the cell cycles at G0/G1 stage, and induced apoptosis (Fig. 5). These results suggest that $p57^{Kip2}$ not only effect on the cell cycles and proliferation but also involve to the tumor metastasis in prostate cancer.

- We will further characterize the functional role and mechanism of $p57^{Kip2}$ in prostate cancer cellular processes by Western Blot, Immunoprecipitation, and CDKs activity assay etc.

- We have recombine $p57^{Kip2}$ stable transfected prostate cancer cells (LNCaP-p57) with rUGM cells to perform tissue recombinant experiments. Histological examination and molecular biological evaluation of tissue samples have been performed by H&E staining and immunohistochemistry staining (Fig. 6).

Fig. 6.

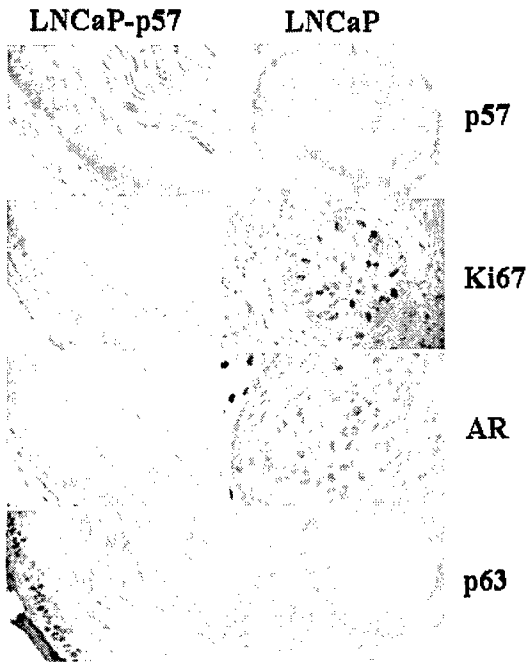

Fig. 6: When the LNCaP cells with an integrated $p57^{Kip2}$ expression vector were recombined with rUGM and grafted underneath the renal capsules of a male athymic mouse host (2 months), the LNCaP tumors transformed to well differentiated squamous tumors and showed increased keratin synthesis (Fig. 6). These results suggest that $p57^{Kip2}$ not only involve to the proliferation, but also involve to the differentiation of LNCaP cells.

Interestingly, the increased expression of $p57^{Kip2}$ induced the p63 expression in LNCaP cells. It has been reported that p63 involved with the tumor metastasis in lung cancer. We will do the further study to conform and understand the relationship between the $p57^{Kip2}$ and p63 in prostate cancer progression.

However, when we combined another $p57^{Kip2}$ stable transfected LNCaP cell line (which expresses higher level of $p57^{Kip2}$, compared that with the first $p57^{Kip2}$ integrated LNCaP cell line) with rUGM and grafted underneath the renal capsules of the male nude mice, there were no detectable LNCaP tumors formed until two months after grafting (0/5).

Reportable Outcomes

Publications

N/A

Abstracts

Renjie Jin, Yongqing Wang, Mingfang Ao, Simon W. Hayward and Robert J. Matusik. $p57^{Kip2}$ is down-regulated in prostate cancer and overexpression inhibits proliferation and tumorigenesis of prostate cancer cells. *Society for Basic Urologic Research Fall 2004 Meeting*, Savannah, GA, December 2004.

Renjie Jin, Yongqing Wang, Mingfang Ao, Simon W. Hayward and Robert J. Matusik. $p57^{Kip2}$ is down-regulated in prostate cancer and overexpression inhibits proliferation and tumorigenesis of prostate cancer cells. *Host-Tumor Interactions Program & Department of Cancer Biology 4th Annual Joint Retreat* November 2004. (**2nd Place Award in Oral Presentation**).

Conclusions

These results suggest that decreased expression of $p57^{Kip2}$ occurs frequently in human prostate cancer even early in PIN lesion and $p57^{Kip2}$ overexpression contributes to the downregulation of cell proliferation and tumorigenesis and progression. Thus, $p57^{Kip2}$ is an important gene in prostate cancer tumorigenesis and progression.

References

N/A

Appendices

CKI: Cyclin-dependent kinases inhibitor.
CDK: Cyclin-dependent kinases.
Dox: Doxycycline
TetR: Tetracycline Repressor
RT-PCR: Reverse Transcription- Polymerase Chain Reaction
rUGM: rat urogenital mesenchyme.
PIN: prostatic intraepithelial neoplasia.